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14. ABSTRACT
Determine the thermostability and thermoactivity of the individual complex components. Study the temperature dependence of whole complex assembly using analytical ultracentrifugation and laser light intensity fluctuation spectroscopy. Investigate the post-translational modification of the E2 component: that is, the lipoylation of the specific lysine in the lipoyl domain. This involves cloning of the genes for the relevant lipoylation enzymes, and characterisation of the protein products.

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Statement of objectives in grant proposal

Thermoplasma acidophilum 2-oxoacid dehydrogenase complex

1. Determine the thermostability and thermoactivity of the individual complex components.
2. Study the temperature dependence of whole complex assembly using analytical ultracentrifugation and laser light intensity fluctuation spectroscopy.
3. Investigate the post-translational modification of the E2 component: that is, the lipoylation of the specific lysine in the lipoyl domain. This involves cloning of the genes for the relevant lipoylation enzymes, and characterisation of the protein products.
4. Study the temperature dependence of whole complex activity and substrate channelling. This includes the phenomenon of active-site coupling.
5. Correlate the data from 1-4 with the investigation of the dynamics of the complex by electron spin resonance.

Aeropyrum pernix and *Pyrobaculum aerophilum*

1. Extend our studies to the complex from one of these hyperthermophilic archaea, both of which grow close to 100°C.

Thermoplasma acidophilum 2-oxoacid dehydrogenase complex

Assembly of the 2-oxoacid dehydrogenase multienzyme complex

We have cloned and expressed the four *Tp. acidophilum* genes that are thought to encode the components of a 2-oxoacid dehydrogenase complex:

- E1α and E1β genes were expressed in *E. coli* to give a soluble $\alpha_2\beta_2$ active enzyme that catalysed the decarboxylation of the branched-chain 2-oxoacids and pyruvate.
- E2 was similarly expressed and Lys42 was found by tryptic mass spectrometry to be post-translationally lipoylated by the *E. coli* host. An E2 assay was developed and the recombinant protein was shown to be catalytically active.
- Sedimentation analysis and dynamic light scattering demonstrated that the E2 core of the complex self-assembled into a 24-mer.
- E3 was also expressed in *E. coli*, found to contain a full complement of FAD and was shown to be active.

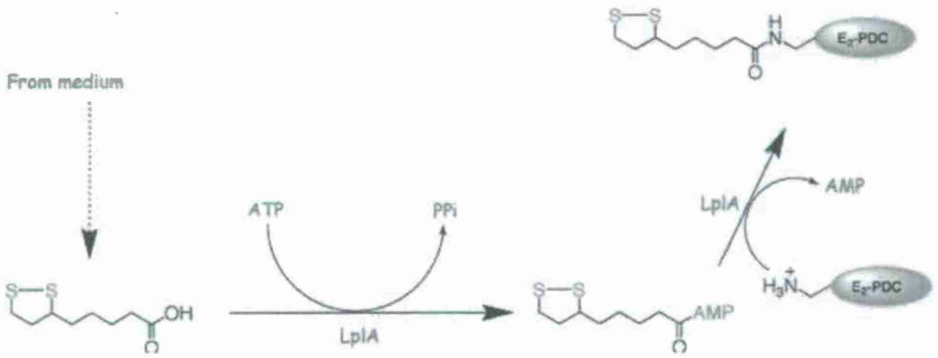
The recombinant enzymes were his-tagged and purified by Nickel-affinity chromatography. They self-assembled into a large (M_r = 5 x 10^6) multienzyme complex, which was shown to catalyse the oxidative decarboxylation of branched-chain 2-oxoacids and pyruvate to their corresponding acyl-CoA derivatives.

Our data constitute the first evidence that the archaea can possess a functional 2-oxoacid dehydrogenase complex.

The archaeal lipoylation system

To produce a functional 2-oxoacid dehydrogenase complex *in vivo*, *Tp. acidophilum* would need to possess the enzymes to lipoylate the E2 component [in the experiments reported above with heterologously-expressed recombinant enzymes, the *E. coli* host carried out the lipoylation].

Blast searches of the *Tp. acidophilum* genome sequence revealed two genes whose protein products showed significant sequence identity to the two domains of the single LplA protein of bacterial systems, which catalyses the reactions:



We have cloned and heterologously expressed the two genes from *Tp. acidophilum* [termed LplA and CTD (C-terminal domain)] and demonstrated that both recombinant proteins are required for the lipoylation of the E2 component, and that the two proteins associate together to carry out this post-translational modification. Moreover, we have preliminary evidence that lipoylation occurs *in vivo* in *Tp. acidophilum*.

This is the first report of the lipoylation machinery in the Archaea, which is unique in that the catalytic activity is dependent on two separate gene products.

Structural studies

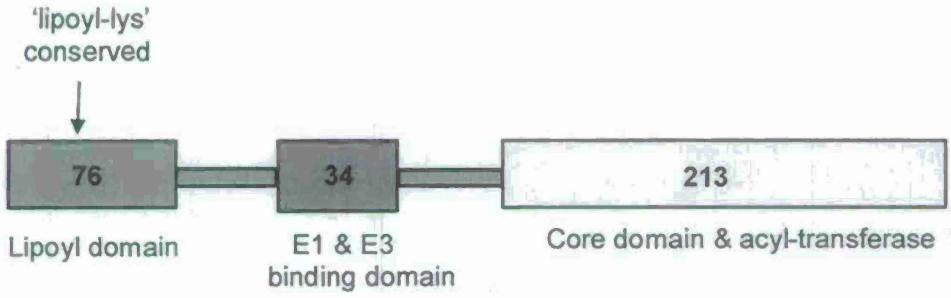
(A) LplA -CTD

We have co-crystallised the active LplA-CTD complex and determined its structure to 2.7Å resolution. It is not yet known if the 2 gene products carry out the 2 separate reactions of the lipoylation process (see above: adenylation of lipoic acid and transfer of the lipoyl group to the E2 lysine side chain), but plans to soak the LplA-CTD crystals with lipoyl-AMP and various analogues may help us to decipher the catalytic mechanism.

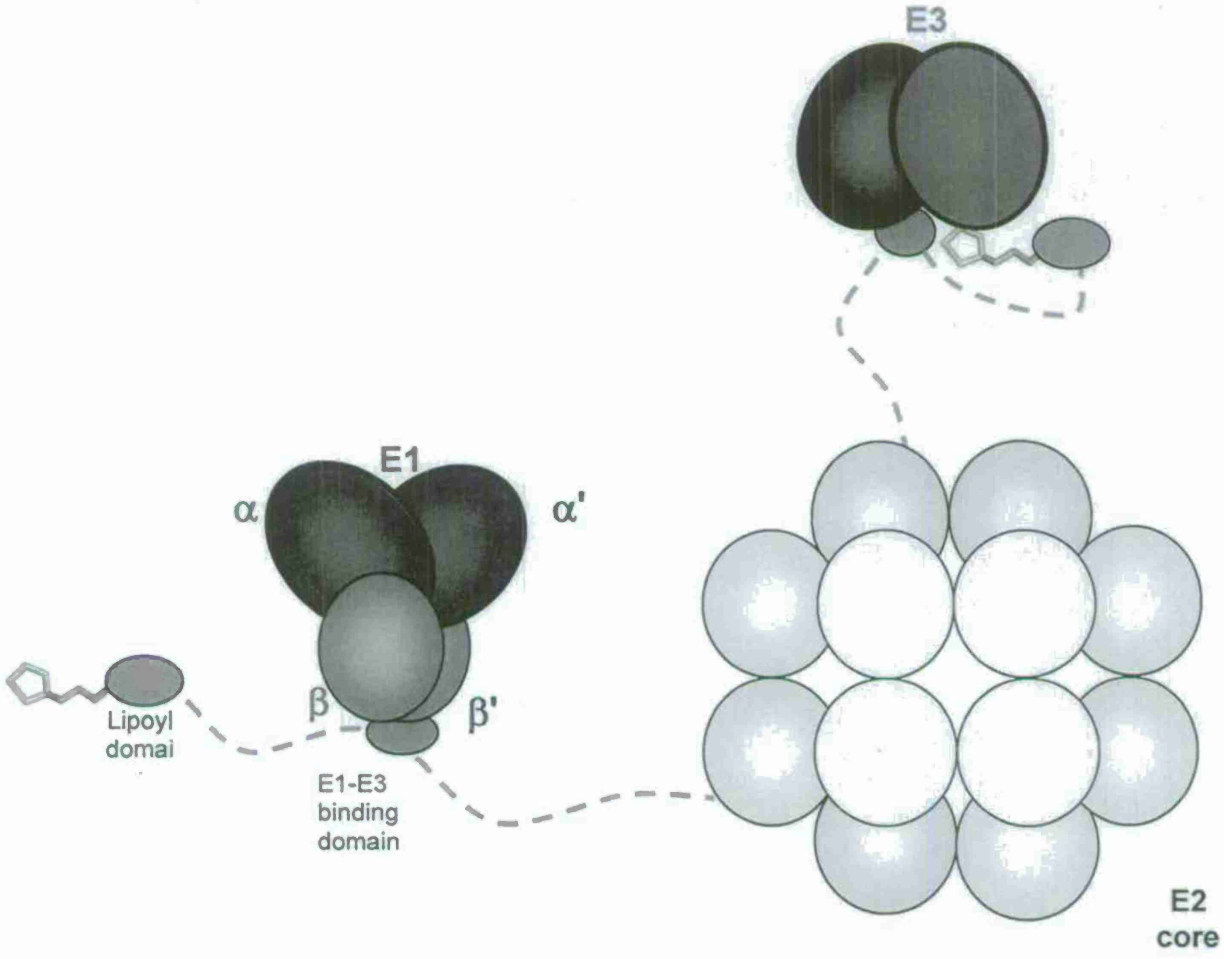
Further experiments are planned to investigate the interaction of LplA-CTD with the E2 lipoyl-domain.

(B) E2 core of the complex

Sequence alignments and structural predictions of the *Tp. acidophilum* E2 core protein show that its domain structure is very similar to that found in bacterial and eukaryotic OADHCs:



Thus, a schematic of the complex assembly might be viewed as:



We are carrying out crystallisation trials with the recombinant $E1\alpha_2\beta_2$ and the E3 homodimer. We have also subcloned the 3 E2 domains: the E2 catalytic domain, the lipoyl domain and the E1-E3 binding domain.

We have shown that the E2 catalytic domain:

- Assembles into a 24 mer
- Is catalytically active in the reverse reaction:

  Acetyl-CoA + dihydrolipoamide ⟶ acetyl-CoA + lipoamide
- Is stable for 10 min at 90°C, and remains as a 24-mer until at least 85°C.

We have crystals of this E2 catalytic domain that diffract to 4Å, and are currently attempting to improve these to get a higher resolution structure.

Our aim in these studies is then to obtain detailed structural information that will allow us to probe the basis of the core assembly and its remarkable thermal stability.

These structural studies are the first on an archaeal OADHC.

Active site coupling and dynamics

We have not made any progress in this area, principally because the electronic spin resonance apparatus has been out of commission due to flood damage during the last year of the grant.

The 2-oxoacid dehydrogenase complex from hyperthermophilic archaea

We have expressed the 4 complex component genes from *Aeropyrum pernix* and *Pyrobaculum aerophilum*, both of which grow optimally at 95-100°C.

Pyrobaculum aerophilum

- We have obtained E1α, E2 and E3 as soluble recombinant proteins, but E1β has proved intractable.
- E2 forms a hyperstable 24-mer, and is catalytically active.
- E3 is active and we obtain a full complement of the FAD cofactor by incubation with excess FAD at 90°C.

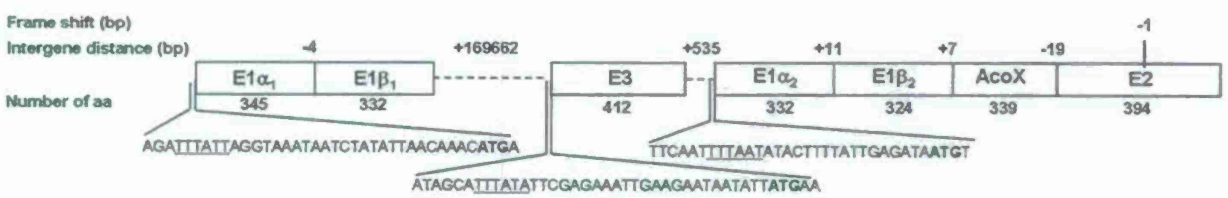
Aeropyrum pernix

- We have obtained E1β, E2 and E3 as soluble recombinant proteins, but E1α has proved intractable.
- E2 forms a hyperstable 24-mer, but no activity has yet been detected.
- E3 is active and we obtain a full complement of the FAD cofactor by incubation with excess FAD at 90°C.

The *P. aerophilum* E1α and the *A. pernix* E1β do not form an active hetero-complex, and therefore we still do not know the substrate specificities of these complexes.

Sulfolobus solfataricus

Therefore, we have turned our attention to the complex from *Sulfolobus solfataricus* (optimal growth at 80-85°C), whose genome contains an unusual OADHC-like operon arrangement and a second copy of E1α and E1β:



That is, unlike the situation in the other aerobic archaea, there is a gene of unknown function (annotated AcoX) between the E1β and E2 genes, E2 has a frame-shift mutation, and the E3 gene is 535 bp upstream of the operon.

We have:

- Successfully obtained recombinant E1α and E1β in soluble form. They associate into an $\alpha_2\beta_2$ tetramer with activity for acetoin, but are inactive with any of the 2-oxoacids (pyruvate, 2-oxoglutarate and the branched-chain 2-oxoacids).
- Cloned the E2 gene, removed the frame-shift mutation by site-directed mutagenesis, and expressed the recombinant protein in a soluble, active form (using the assay described for the *Tp. acidophilum* E2).
- Demonstrated that the E2 forms a 24-mer core and is a hyperstable assembly.

Strangely, we have failed to obtain active E3 recombinant enzyme, despite attempting numerous expression strategies. Currently, we are collaborating with Professor Sonja Albers to express the gene in an homologous *Sulfolobus* system.

Staff

AFOSR grant			
Professor Michael Danson	PI	3 years	full time
Dr David Hough	Co-PI	3 years	full time
Dr Karl Payne	Post-doctoral researcher	3 years	full time
Dr Claire Spreadbury	Technician	1 year	0.5
Jodie Gibson	Technician	2 years	0.5

Associated researchers	
Dr Mareike Posner	University of Bath Studentship (2005-2008) UNESCO L'Oréal International Fellowship (2009-2011)
Nia Hughes	Biotechnology & Biological Sciences Research Council (UK) Studentship (2008-2012)

Publications and presentations

Peer-reviewed publications

[1] Heath, C., Posner, M.G., Aass, C.H., Upadhyay, A., Scott, D.J., Hough, D.W. & Danson, M.J. (2007) *FEBS J.* 274, 5406–5415. **The 2-oxoacid dehydrogenase multi-enzyme complex of the archaeon *Thermoplasma acidophilum* – recombinant expression, assembly and characterization.**

[2] Posner, M.G., Upadhyay, A., Bagby, S., Hough, D.W. & Danson, M.J. (2009) *FEBS J.* 276, 4012-4022.
A unique lipoylation system in the archaea: lipoylation in *Thermoplasma acidophilum* requires two proteins.

[3] Payne, K.A.P., Hough, D.W. & Danson, M.J. (2009)
FEBS Lett. 584, 1231–1234
Discovery of a putative acetoin dehydrogeanse complex in the hyperthermophilic archaeon, *Sulfolobus solfataricus*.

[4] Daniel, R.M. & Danson, M.J. (2010)
Trends Biochem. Sci. In Press
A new understanding of how temperature affects the catalytic activity of enzymes.

Papers in progress

[1] The crystal structure of the lipoylation enzymes (CTD-Lpl-A) has been determined and this will be submitted in the near future to the *J. Mol. Biol.*

[2] The structural work on the E2 cores from the hyperthermophilic archaea will lead to a peer-reviewed publication, but further work is needed, and is currently in progress, to make a substantial publication.

Published conference abstracts

[1] Danson, M.J., Posner, M.G., Heath, C. & Hough, D.W. (2007) *Proc. Int. Conf. "Thermophiles 2007" (University of Bergen, Norway)* Abstract L29, p. 48. **Multienzyme complexes in central metabolism of thermophilic archaea: discovery, structure and function.**

[2] Payne, K., Heath, C., Posner, M.G., Spreadbury, C., Hough, D.W. & Danson, M.J. (2008) *Proc. Int. Conf. "Extremophiles 2008" (Cape Town, South Africa)* Abstract O34, p. 61
2-Oxoacid dehydrogenase multienzyme complexes in thermophilic archaea.

[3] Hughes, N.L., Scott, D.J., van den Elsen, J., Hough, D.W. & Danson, M.J., (2010)
Proc. Biochem. Soc. Meeting "Experimental Approaches to Protein-Protein Interactions (Sheffield, UK) In Press
The 2-oxoacid dehydrogenase multienzyme complex from thermophilic Archaea.

University and conference invited talks

MJD has given the following presentations on the multienzyme complex research carried out in the AFOSR grant.

[1]	International Conference "Thermophiles 2007", Bergen, Norway	Sep 2007
[2]	Department of Biological Sciences, University of Kuwait, Kuwait	Oct 2007
[3]	University of the Western Cape, Cape Town, South Africa Int. Workshop at "Institute of Microbial Biotechnology & Metagenomics"	Sep 2008
[4]	International Conference "Thermophiles 2009", Beijing, China	Aug 2009
[5]	Int. Conference "Protein Engineering and Design", Regensburg, Germany	Sep 2009
[6]	Institute of Structural & Molecular Biology, University College London, UK	Dec 2009
[7]	Dept of Biol. & Biochemistry Research Conference, University of Bath, UK	Feb 2010
[8]	Dept of Molecular Biology & Biotechnology, University of Sheffield, UK	Feb 2010
[9]	School of Physics and Astronomy, University of Leeds, UK	Apr 2010

MULTI-ENZYME COMPLEXES IN THE THERMOPHILIC ARCHAEA

The effects of temperature on stability, catalysis and enzyme interactions in a multi-component system

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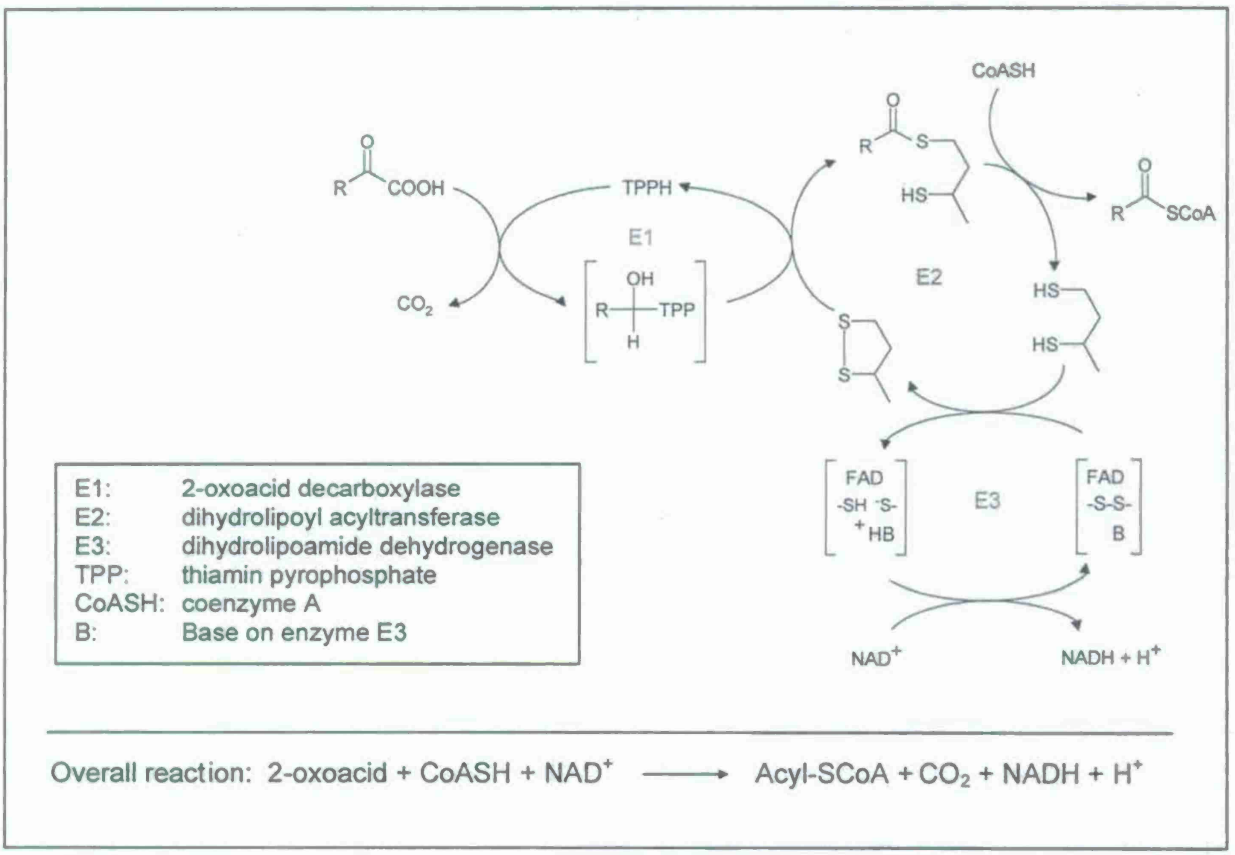
FINAL REPORT

Essential background information

The 2-oxoacid dehydrogenase complexes comprise multiple copies of 3 component enzymes, resulting in multienzyme particles of up to 100 polypeptide chains, through which substrate is channelled via a complex but efficient mechanism of active-site coupling. The individual components and the overall catalytic mechanism of the complex are illustrated below:



E1:	2-oxoacid decarboxylase
E2:	dihydrolipoyl acyltransferase
E3:	dihydrolipoamide dehydrogenase
TPP:	thiamin pyrophosphate
CoASH:	coenzyme A
B:	Base on enzyme E3

Overall reaction: 2-oxoacid + CoASH + NAD^+ ⟶ Acyl-SCoA + CO_2 + NADH + H^+

These complexes have never been found as active enzymes in any of the archaea, but our discovery of genes in the aerobic archaea that appear to encode the components of such complexes formed the basis of the AFOSR grant.